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Natural Mutations in the Receptor Binding Domain of Spike Glycoprotein Determine the Reactivity of Cross-Neutralization between Palm Civet Coronavirus and Severe Acute Respiratory Syndrome Coronavirus[▽]

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The severe acute respiratory syndrome (SARS) outbreak of 2002 and 2003 occurred as a result of zoonotic transmission. Coronavirus (CoV) found in naturally infected palm civet (civet-CoV) represents the closest genetic relative to SARS-CoV, but the degree and the determinants of cross-neutralization among these viruses remain to be investigated. Studies indicate that the receptor binding domain (RBD) of the SARS-CoV spike (S) glycoprotein contains major determinants for viral entry and neutralization. We aim to characterize the impact of natural mutations within the RBDs of civet-CoVs on viral entry and cross-neutralization. In this study, the S glycoprotein genes were recovered from naturally infected civets in central China (Hubei province), extending the geographic distribution of civet-CoV beyond the southeastern province of Guangdong. Moreover, pseudoviruses generated in our laboratory with four civet S genes, each with a distinct RBD, infected cells expressing human receptor angiotensin-converting enzyme 2, but with 90 to 95% less efficiency compared to that of SARS-CoV. These four civet S genes were also constructed as DNA vaccines to immunize mice. Immunized sera elicited against most civet S glycoproteins displayed potent neutralizing activities against autologous viruses but were much less efficient (50% inhibitory concentration, 20- to 40-fold) at neutralizing SARS-CoV and vice versa. Convalescence-phase sera from humans were similarly ineffective against the dominant civet pseudovirus. Our findings suggest that the design of SARS vaccine should consider not only preventing the reemergence of SARS-CoV but also providing cross-protection, thus interrupting zoonotic transmission of a group of genetically divergent civet CoVs of broad geographic origin.

Severe acute respiratory syndrome (SARS) is an emerging infectious disease caused by a novel coronavirus (CoV) variant, SARS-CoV (20, 22). During the epidemic from 2002 through 2003, SARS-CoV was highly transmissible in humans, which led to a total of 8,437 infections and 813 (9.6%) deaths. Although the SARS epidemic was successfully contained by July 2003, it is only prudent that the scientific and public health communities proceed with the expectation that SARS will return in the future.

Continuous efforts have been made toward the development of a prophylactic vaccine to prevent a possible reemerging epidemic (1, 5, 8, 28). Two recent studies have identified horseshoe bats (genus *Rhinolophus*) as a potential animal reservoir of SARS-CoV-like viruses (13, 16). However, these studies also indicate that there are significantly low levels of sequence similarity (79 to 80%) between CoV in bats (bat-CoV) and SARS-CoV in genes encoding the viral spike (S) glycoprotein (13). Due to this difference, the S glycoprotein of bat-CoV

cannot interact with human receptor angiotensin-converting enzyme 2 (ACE2), which is essential for cross-species transmission (14, 15). Therefore, these bats are unlikely to be the direct transmitters of SARS-CoV to humans. To this end, the Himalayan palm civet (*Paguma larvata*) probably serves as a critical immediate host, given the high level of genetic similarity (~99%) between S glycoproteins for CoV in civets (civet-CoV) and those of SARS-CoV (6, 9, 12). Recent S glycoprotein structure and mutagenesis studies indicate that the zoonotic transmission may require key amino acid mutations in the receptor binding domain (RBD) of S glycoprotein which determines the viral entry mediated by human ACE2 (4, 14, 17, 21, 29). To date, whether natural mutations within the RBDs of genetically divergent civet-CoVs affect viral entry and cross-neutralization remains elusive.

Several studies have provided convincing evidence that neutralizing antibodies (NAbs) play a central role in the prevention of SARS-CoV infection (2, 5, 24, 25, 28). A high level of NAbs can be readily elicited using various forms of vaccines that express the S glycoprotein or just the RBD (1, 5, 11, 18, 28). Importantly, protective immunity was achieved using several of these vaccines in mouse or monkey models (5, 8, 24, 31). However, whether the NAbs induced by these vaccines could

confer protection against genetically divergent civet-CoVs remains unclear. In this report, we evaluated four S glycoproteins with distinct RBD sequences representing 38 civet-CoVs identified in the Guangdong and Hubei provinces of China. We characterized the impact of these distinct changes on viral entry efficiency and susceptibility to autologous and heterologous NAbs in immunized animals and convalescent-phase sera of SARS patients.

MATERIALS AND METHODS

Sequence analysis of S genes of SARS-CoVs and civet-CoVs. Reverse transcription-PCR was used to recover S genes from three Himalayan palm civets found in the central China province of Hubei (GenBank accession numbers DQ514530, DQ514529, and DQ514528). These sequences were compared with those in the S genes of 35 civet-CoVs and of multiple SARS-CoVs deposited in GenBank. Nucleotide sequences were aligned, and a phylogenetic tree was generated by the neighbor-joining method using computer software implemented in the CLUSTALX 1.81 program (26). The branch significance was analyzed by bootstrap with 1,000 replicates. The tree was plotted using the TreeView program (19).

Generation of full-length S genes with distinct RBD sequences. With the codon-optimized, full-length, SARS-CoV S gene as the basis (5, 30), we constructed four full-length S genes with distinct civet-CoV RBD sequences, namely hu/ciGD1, ciSZ, ciHB, and ciGD2 in the pcDNA3.1 vector using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Each plasmid was confirmed by sequence analysis.

Expression of full-length civet-CoV S glycoprotein. The full-length civet-CoV S gene in the plasmid pcDNA3.1-SARS-CoV-S, pcDNA3.1-hu/ciGD1-S, pcDNA3.1-ciSZ-S, pcDNA3.1-ciHB-S, or pcDNA3.1-ciGD2-S was transfected into the 293T cells, and the expression level was determined by Western blot assay as we previously described (29).

Immunizations of mice with full-length S glycoprotein expression vectors. Five groups (three mice per group) of 6- to 8-week-old female BALB/c mice (Charles River Laboratories) were immunized with 20 μg of plasmid DNA in 50 μl saline by in vivo electroporation (ICHOR Med-System, San Diego, CA). Mice were vaccinated twice at weeks 0 and 3, and sera were collected at week 5. Animal experiments were approved by and conducted in the Laboratory Animal Research Center at Rockefeller University.

Viral entry assay. The pseudovirus was generated by cotransfecting (SuperFect; QIAGEN) 293T cells with human immunodeficiency virus type 1 pNL4-3Luc⁺Env⁻Vpr⁻ backbone and one of the above-mentioned, full-length S gene expression plasmids. Viral supernatant was collected 48 h posttransfection and frozen at −150°C. The viral p24 antigen was measured using the human immunodeficiency virus type 1, p24 antigen, enzyme immunoassay (Beckman Coulter, Fullerton, CA). The entry assay was performed as we previously described (29).

Neutralization assay. A pseudovirus-based neutralization assay was used to determine the susceptibility of the civet-CoV S glycoprotein to serum neutralization from immunized animals and infected humans (5, 29). The neutralizing activity of heat-inactivated sera (56°C, 30 min) was determined by mixing 5 ng of pseudovirus (in 30 μl) with diluted serum (in 30 μl) at 37°C for 1 h. A detailed procedure has been described previously (29). Convalescence-phase sera from SARS patients were collected from people who recovered from SARS-CoV infection at Peking Union Medical College Hospital in Beijing. These samples were anonymously provided for this study.

RESULTS

Identification of three civet-CoVs in the central China province of Hubei. Previous studies have identified 35 civet-CoVs in Guangdong where the SARS epidemic of 2002 through 2003 originated. In this study, we were able to recover S genes from three civets found in farms in the central China province of Hubei, which is geographically distant from the province of Guangdong. This is the first report of the identification of naturally infected civets outside the Guangdong province. The discovery of civet-CoVs in the Hubei province should not be a surprise as SARS-CoV-like viruses were recently found in
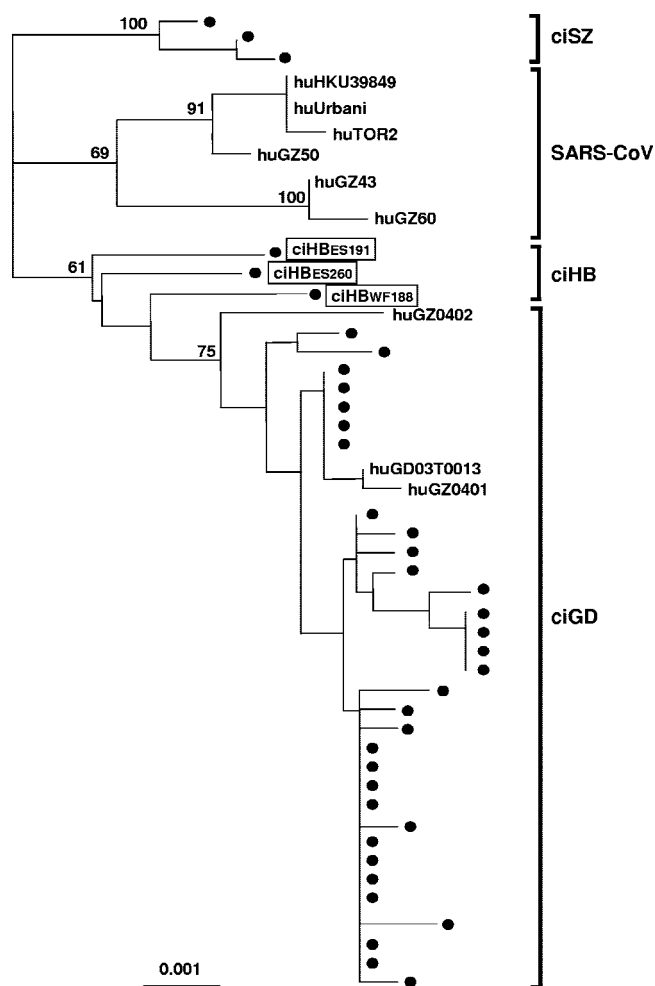

FIG. 1. Phylogenetic neighbor-joining tree for civet-CoV S1 sequences. Three new Hubei strains are boxed. The reference sequences were obtained from GenBank. Human strains are spelled out, whereas civet viruses are presented as black dots. The GenBank accession numbers of the civet-CoVs include AY304486, AY304488 and -9, AY572034 and -5, AY572037 and -8, AY627044, AY627046 to 8, AY613948 and -9, AY613950 to 2, AY687354 and -6, AY687359, AY687360, AY686863 and -4, AY687361 to 9, and AY687370 and -2 (12, 23, 27). The horizontal branch is drawn in accordance with their relative genetic distances. The vertical lines provide clarity to the tree presentation. Bootstrap values of 1,000 replicates are labeled on the major branches. ci, civet; hu, human; SZ, Shenzen, GD, Guangdong; HB, Hubei.

a bat species in the same province (16). Based on the phylogenetic analysis of S genes, the three new viruses are relatively divergent from one another and also from the viruses previously identified (Fig. 1). This finding indicates that the spread of civet-CoVs in the Hubei province is unlikely to be recent. Based on the genetic distance, it is likely that there are three major clusters of civet-CoVs (named ciSZ, ciHB, and ciGD) in the phylogenetic tree (Fig. 1). Since three viruses found in the Hubei province are more closely related to ciGD, the dominant cluster of civet-CoVs, than to ciSZ or SARS-CoV, these civet viruses probably shared a common ancestor. However, whether viral variants within the dominant cluster of civet-CoVs harbor any distinct biological functions remains unclear.

Six major amino acid changes were found in the RBD region

TABLE 1. Amino acid differences in the RBD of S glycoprotein of SARS-CoV and civet-CoVs

Virus	Residue at position						Number[a]	Location	Reference(s)
	344	360	472	479	480	487			
SARS-CoV	K	F	L	N	D	T	NA	Worldwide	6, 20, 22
ciSZ	R	S	L	K	D	S	3/38	Guangdong	9
hu/ciGD1[b]	R	S	P	N	G	S	8/38	Guangdong	12, 23, 27
ciGD2	R	S	P	R	G	S	25/38	Guangdong	12, 23, 27
ciHB	R	F	P	R	G	S	2/38	Hubei	This study

[a] Values are expressed as the number of civet viruses found in each group/total number of civet viruses. NA, not applied.
[b] There are three additional human isolates in this group.

of S glycoprotein at positions 344, 360, 472, 479, 480, and 487 (Table 1), and four of these (472, 479, 480, and 487) are located within the receptor binding motif of RBD that makes contacts with the viral receptor ACE2 (14). Using the phylogenetic clusters, we attempted to divide civet-CoVs into ciSZ, ciHB, and ciGD groups for functional analysis (Fig. 1). The ciSZ group represents the earliest civet-CoVs obtained in the Shenzhen city of the Guangdong province (9). The ciHB group represents civet-CoVs from a previously unrecognized geographic location. ciGD represents the major groups of civet-CoVs in the province of Guangdong. Considering that there is a critical amino acid difference at position 479, we further proceeded to divide ciGD into hu/ciGD1 and ciGD2 (Table 1). The hu/ciGD1 represents eight civet-CoVs and three human isolates (huGD03T0013, huGZ0401, and huGZ0402) (Fig. 1). One virus from the province of Hubei, $ciHB_{ES260}$, was included in hu/ciGD1 because it has the same RBD sequence as other viruses in this group. Ultimately, ciGD2 represents 25 civet-CoVs and therefore is the most dominant form of RBD (Fig. 1 and Table 1).

The natural variations in the RBD of civet-CoV significantly reduce the entry efficiency of pseudoviruses. We replaced only the RBD of the SARS-CoV S glycoprotein with each of the four civet RBDs (29). Of note, except for ciSZ, the resulting civet-CoV S glycoproteins did not contain amino acid changes outside their RBDs relative to that in the corresponding region of SARS-CoV. ciSZ contains an additional 261K mutation to be consistent with previous studies (9, 17). These S glycoproteins with distinct RBDs were expressed at comparable levels in 293T cells as determined by the Western blot assay (Fig. 2A). To determine the impact of these mutations within the RBDs on mediating viral entry, we examined the entry efficiency of pseudotyped virus bearing each of the four S glycoproteins into the HEK293T-ACE2 cells. We found that all civet pseudoviruses were able to infect the target cells but with a 90 to 95% reduction in entry efficiency (Fig. 2B). In particular, ciSZ is the weakest virus; it is about two- to threefold less efficient than other civet pseudoviruses. We conducted parallel studies with HEK-293T cells and found that none of the pseudoviruses infected these cells (data not shown). These results suggest that all civet-CoVs use human ACE2 as an entry receptor in a specific but much less efficient manner, which is consistent with a previous finding with the civet-CoV_{SZ3} (17).

Civet pseudoviruses are preferentially neutralized by antibodies induced by autologous DNA vaccines. Considering that civet pseudoviruses enter HEK293T-ACE2 cells in a less ef-

fective way, we sought to determine whether this property would interfere with the susceptibility of these viruses to NAbs induced by vaccines. Four groups of mice were immunized with DNA vaccines, including pcDNA3.1-hu/ciGD1-S, pcDNA3.1-ciSZ-S, pcDNA3.1-ciHB-S, and pcDNA3.1-ciGD2-S, by using an in vivo electroporation technique (29). As a control, the DNA vaccine for SARS-CoV (pcDNA3.1-OPT9) was tested in parallel. The animal immune sera were tested for the neutralization of autologous and heterologous pseudoviruses. Consistently, the autologous DNA vaccine induced the highest level of NAbs to each corresponding pseudovirus. For example, the autologous serum DNA-S131 neutralized the SARS-CoV pseudovirus with the highest efficiency (Fig. 3, top row, left panel). Similarly, serum ciSZ3-112 neutralizes the ciSZ virus
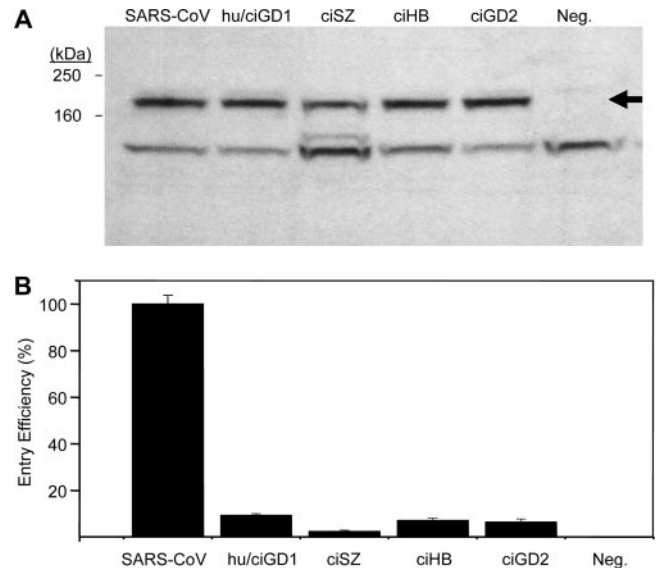

FIG. 2. Expression and functional analysis of S glycoproteins derived from SARS-CoV and civet-CoVs. Similar levels of expression of each S glycoprotein (SARS-CoV, ciSZ, hu/ciGD1, ciGD2, or ciHB) are detected in Western blots stained with a polyclonal antibody to the first 400 amino acids of the N terminus (A). The arrow indicates the position of S glycoprotein. The vector cell lysate was used as a negative control. The viral entry efficiency was determined in human HEK293T-ACE2 cells (B). Five nanograms (measured by p24) of each pseudovirus was used to infect target cells. Luciferase activity was measured 72 h postinfection and normalized with SARS-CoV infection (100%). Five replicates were tested in each experiment. The average values and standard error bars are presented. The experiment was repeated three times.
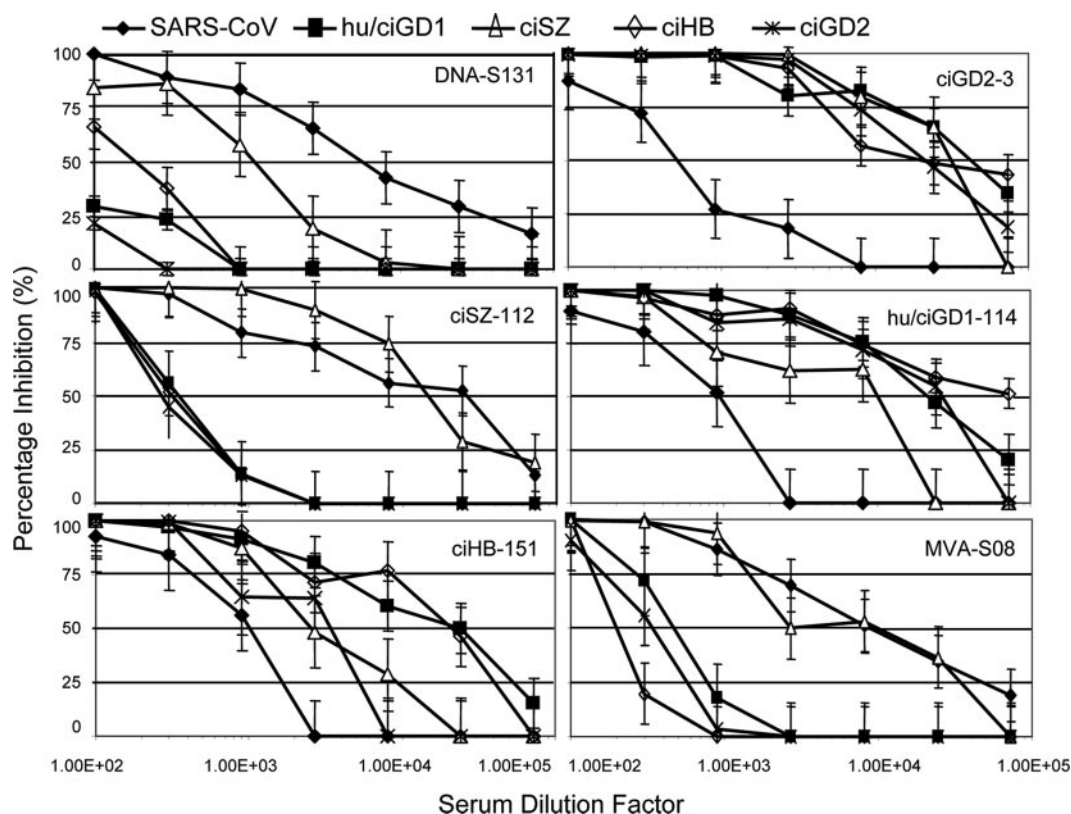
FIG. 3. Neutralization and cross-neutralization of SARS-CoV and civet-CoVs. Six groups of immune sera were tested against four civet pseudoviruses and an SARS-CoV pseudovirus. Five groups of sera were from mice immunized by electroporation with pcDNA3.1-OPT9 (top left panel), pcDNA3.1-ciSZ3-S (middle left panel), pcDNA3.1-ciHB-S (bottom left panel), pcDNA3.1-ciGD2-S (top right panel), and pcDNA3.1-hu/ciGD1-S (middle right panel) DNA vaccines, respectively. The sixth group of sera was from mice immunized with the ADS-MVA vaccine (bottom right panel). Sera were diluted from 1:100 to 1:72,900 and tested to neutralize 5 ng (measured by p24) of each pseudovirus in human HEK293T-ACE2 cells. Duplicates were tested in each experiment. The average values from each group are presented. Standard error bars are included. The experiment was repeated twice with similar results.

with the highest efficiency (Fig. 3, middle row, left panel), as did the other autologous sera. Moreover, the magnitude of the NAb levels against each pseudovirus was comparable with 50% inhibitory concentration (IC_{50}) values around the serum dilution of 1:10^4. These findings indicate that the reduced level of receptor usage of civet pseudoviruses does not affect viral susceptibility to antibody neutralization.

Natural variations in the RBDs significantly contribute to the cross-neutralization resistance between civet-CoV and SARS-CoV. In comparison to autologous serum neutralization, an important finding was that the serum DNA-S131 neutralized most heterologous civet pseudoviruses rather poorly, with a reduced IC_{50} of around 32-fold (ciHB) to about 100-fold (hu/ciGD1 or ciGD2) ($P < 0.01$) (Fig. 3, top row, left panel). Only the ciSZ pseudovirus remained sensitive to the DNA-S131 serum with a minor reduction in IC_{50} (fivefold). Conversely, the sera elicited against most civet RBDs neutralized SARS-CoV ineffectively with similarly reduced IC_{50} values of around 21- (ciHB-151), 24- (hu/ciGD1-114), and 42-fold (ciGD2-3) ($P < 0.01$) (Fig. 3, bottom left, middle right, and top right panels). Again, only serum against ciSZ neutralized SARS-CoV with a minor reduction in IC_{50} (sixfold) (Fig. 3, middle left panel). These data suggest that the common neutralizing epitopes shared by SARS-CoV and ciSZ3 are quite

different from those harbored by the other three civet-CoVs. Interestingly, the civet-CoVs may share some common neutralizing epitopes as well. Serum ciGD2-3 neutralized all four civet pseudoviruses very effectively but with an IC_{50} that was around 41-fold reduced against SARS-CoV ($P < 0.01$) (Fig. 3, top right panel). Similar findings were obtained when additional mouse sera were tested for each group. We therefore believe that the six nonsynonymous differences in the RBDs have a great impact on the neutralization susceptibility profile between SARS-CoV and ciGD2 (Table 1). Since there is just one amino acid difference between ciGD2 and hu/ciGD1 (R479N) or between ciGD2 and ciHB (S360F) (Table 1), hu/ciGD1 and ciHB display neutralization profiles closer to ciGD2 than to ciSZ. On the other hand, the two amino acids harbored by SARS-CoV and ciSZ (472L and 480D) likely contribute to a major neutralizing epitope that they share. If the differences in NAb profiles between ciSZ and ciGD2 were a result of viral evolution in civets, we speculate that this animal species might have harbored civet-CoVs for some time.

Civet pseudoviruses are resistant to NAbs induced by a different form of SARS vaccine. We previously demonstrated that a modified vaccinia Ankara-based vaccine (ADS-MVA) induces protective immunity in Chinese rhesus monkeys against a pathogenic SARS-CoV challenge (5). ADS-MVA
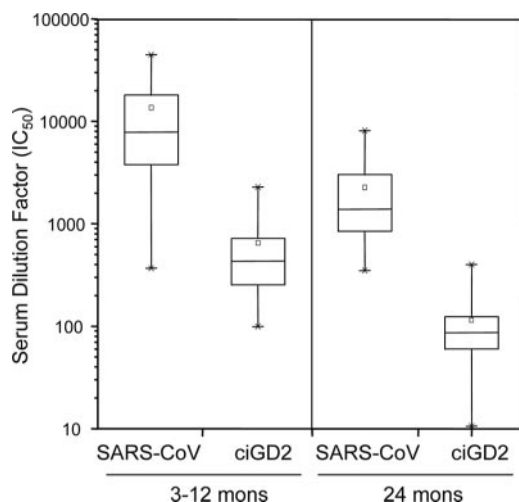
FIG. 4. Resistance of ciGD2 pseudovirus to neutralization by human convalescent-phase sera of SARS patients. In comparison to the SARS-CoV pseudovirus, the ciGD2 pseudovirus displayed greatly reduced neutralization susceptibility to 20 human convalescent-phase sera collected 3 to 12 months p.r. at an average of 41-fold, which is highly significant (signed rank test; $P < 0.005$) (left panel). Ten additional serum samples collected at 24 months p.r. (right panel) show further reductions in NAb levels against both pseudoviruses. Consistently, ciGD2 pseudovirus displayed significantly less neutralization to these 10 convalescent-phase sera than did SARS-CoV pseudovirus, which is also highly significant ($P < 0.005$). The top and bottom of each rectangular box denote the 75th and 25th percentiles, respectively, with the median shown inside the box. Horizontal bars extending from each box represent the 99th and 1st percentiles. Duplicates were tested in each experiment. The average values are presented for each experiment. The experiment was repeated twice.

contains the S gene of SARS-CoV$_{HKU39849}$ in its deletion III region. The expression of the S gene is driven by a vaccinia-specific early and late synthetic promoter (5). We wanted to determine whether the NAbs induced by ADS-MVA would neutralize civet pseudoviruses in a different way. Serum MVA-S08, which was induced by ADS-MVA, was tested against the same panel of pseudoviruses (Fig. 3, bottom right). We found that the serum MVA-S08 neutralized most heterologous civet pseudoviruses, including ciGD1, ciGD2, and ciHB, poorly with reduced IC$_{50}$ values of 12-, 15-, and 26-fold, respectively. The neutralization profile of MVA-S08 is similar to that observed for serum DNA-S131 (Fig. 3, top left panel). Additional mouse sera raised by the ADS-MVA vaccine gave similar results. Our data suggest that both DNA- and MVA-based SARS vaccines have likely induced similar NAbs that are considerably less sufficient to neutralize most civet-CoVs.

Civet-CoVs are significantly less susceptible to NAbs in the convalescence-phase sera of SARS patients. Considering that the vaccine-induced NAbs may not necessarily represent NAbs generated during the natural course of SARS-CoV infection, we further tested convalescence-phase sera collected from people who were infected in mainland China during the epidemic period of 2002 through 2003. These sera were collected from patients 3 to 12 or 24 months postrecovery (p.r.) from the illness. In comparison to the SARS-CoV pseudovirus, the ciGD2 pseudovirus displayed greatly reduced neutralization susceptibility to 20 human convalescence-phase sera collected

3 to 12 months p.r. at an average of 41-fold (range, 2- to 156-fold), which is highly significant (signed rank test; $P < 0.005$) (Fig. 4, left panel). Furthermore, we tested another 10 sera collected at 24 months p.r. (Fig. 4, right panel). We observed a further reduction in NAb levels against both the SARS-CoV and ciGD2 pseudoviruses. Consistently, the ciGD2 pseudovirus displayed significantly less neutralization susceptibility than did the SARS-CoV pseudovirus, which is also statistically significant ($P < 0.005$). Of note, two individuals have probably lost NAbs against ciGD2 as no neutralization activity was detected at the serum dilution of 1:10. With the marked wane of NAb titer over time, these people will probably become vulnerable to remerging SARS-CoV or civet-CoV infection.

DISCUSSION

We report here our findings in addressing the impact of natural mutations in the RBD of S glycoprotein on viral entry and cross-neutralization between SARS-CoV and civet-CoV. We found that amino acid changes in the RBDs, which resemble naturally occurring civet-CoVs, reduce the level of viral entry into human ACE2 cells significantly. Unexpectedly, these changes confer remarkable resistance to NAbs generated by S glycoprotein-based SARS-CoV vaccines. Furthermore, we found that convalescent-phase sera collected from SARS patients are significantly less efficient at neutralizing the dominant group of civet-CoV. Since we also found three naturally infected civets in Hubei, which extends the geographic distribution of civet-CoVs beyond the Guangdong province, our findings pose challenges to the prevention of remerging SARS-CoV or zoonotic transmissions of SARS-CoV-like viruses.

The sequence diversity in the RBD of civet-CoV S glycoprotein determines the level of human receptor ACE2-mediated viral entry, which is likely critical for zoonotic transmission. ACE2 is a functional cellular receptor for SARS-CoV (15). It was suggested that the adaptation of civet-CoV to human ACE2 was a critical determinant for the severity of the SARS epidemic of 2002 and 2003. This is because the civet-CoV (ciSZ) isolated from civets used human ACE2 markedly less efficiently than did the epidemic strain SARS-CoV due to the lack of residues 479N and 487T in S glycoprotein (9, 17). Consistent with these findings, we found that civet-CoVs represented by hu/ciGD1, ciHB, and ciGD2 all utilize human ACE2 in a specific but much less effective way than SARS-CoV does. This finding is probably because none of these civet-CoVs contain 479N and 487T simultaneously (Table 1). Interestingly, hu/ciGD1 contains 479N but not 487T. Since this 479N was found in eight civet-CoVs (Table 1), it is unlikely that this mutation in SARS-CoV is a result of viral adaptation in humans. To this end, the additional mutation 487T has probably played the central role in adapting the use of human ACE2. Nevertheless, the transmission of civet-CoVs to humans is not strictly restricted by this 487T mutation. Three human viruses, GD03T0013, GZ0401, and GZ0402, which are in the group of hu/ciGD1 and do not contain 487T (Fig. 1), caused human infections in the 2003 and 2004 periods despite the fact that infected patients had mild clinical presentations and low transmissibility (12, 23). Since these human viruses are genetically closer to ciGD1 than to SARS-CoV, they likely represent at least one indepen-

dent cross-species event in addition to the one that led to the global SARS epidemic (Fig. 1) (27). As most civet pseudoviruses enter human ACE2 cells with an efficiency similar to that of ciGD1 (Fig. 2B), it is not surprising to find a high prevalence rate among traders who sell civets, an indication of frequent zoonotic transmission (3).

The natural mutations in the RBD of civet-CoVs S glycoprotein determine the level of viral cross-neutralization. It was previously demonstrated that the RBD of SARS-CoV contains the major neutralizing determinants (5, 11, 29). Moreover, a single nonsynonymous mutation in the RBD of SARS-CoV could either abolish the immunogenicity of S glycoprotein for inducing potent NAbs (e.g., R441A) or result in an escape virus to a monoclonal NAb (e.g., P462L) (25, 29). These findings suggest that a limited number of amino acid changes in the viral RBD can substantially alter the profile of NAbs. To this end, although there are only a few amino acid differences in the RBDs of SARS-CoV and civet-CoV$_{SZ3}$, some monoclonal NAbs clearly recognize these two viruses in a different conformational fashion (10). Here, we demonstrate for the first time that the natural mutations in the RBD render most civet-CoVs greatly resistant to NAbs induced by S glycoprotein-based SARS-CoV vaccines. It is possible that these changes might have altered the conformational structure of S glycoprotein for immune recognition. Since the dominant group of civet-CoVs are also resistant to neutralization by convalescence-phase sera collected from SARS patients, our data raise concerns about the conclusion of a recent study stating that the major neutralizing epitopes of SARS-CoV have been apparently maintained during cross-species transmission and that RBD-based vaccines may induce broad protection against both human and animal SARS-CoV variants (10). Since their conclusion was based on findings with civet-CoV$_{SZ3}$, this virus clearly does not represent most civet-CoVs. Up to now, whether the selection pressure of NAb response in humans fostered the emergence of the pathogenic SARS-CoV remained unclear. Given the significantly low level of cross-neutralization between SARS-CoV and most civet-CoVs, we speculate that the immune selection could have played a role in this regard.

A successful vaccine should elicit broad and potent NAbs against SARS-CoV and its related CoVs from animals. To prevent the reemergence of SARS, the development of an effective vaccine remains the top priority. However, the currently developed vaccines target SARS-CoV without much emphasis on its related viruses. Our findings have provided evidence that S glycoprotein-based SARS vaccines are probably not sufficient to prevent zoonotic transmission of most civet-CoVs.

ADDENDUM

While this paper was being revised, an independent study demonstrated that only limited protection was seen in vaccinated senescent mice against the heterologous icGD03-S challenge (7). icGD03-S is equivalent to hu/ciGD1 in this study. To overcome the problem, it is possible to design a combined vaccine by including the S glycoproteins of both SARS-CoV and ciGD2 to provide a broad spectrum of protection (Fig. 3). Such a vaccine will hopefully not only provide protection against the reemergence of SARS-CoV but also eliminate the

chance of civet-CoV adaptation in humans by preventing frequent zoonotic transmission.

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